Epidermal Keratinocyte Production of Interferon-γ Immunoreactive Protein and mRNA Is an Early Event in Allergic Contact Dermatitis

Sarah E. M. Howie,* Roger D. Aldridge,† Eva McVittie,† Rosalyn J. Forsey,*† Catherine Sands,*† and John A. A. Hunter†

Departments of *Pathology and †Dermatology, Edinburgh University Medical School, Teviot Place, Edinburgh EH8 9AG, United Kingdom

Previous work has indicated the importance of cytokine cascades in the induction of contact dermatitis, but there is little information on the cellular localization of cytokines in human skin, particularly during the early phases of the inflammatory response to contact allergens. Using *in situ* hybridization for mRNA and immunocytochemistry on biopsies from a series of 16 patients with known allergic contact dermatitis, we examined the kinetics of early cytokine production after challenge with relevant or irrelevant antigen. We show that epidermal keratinocytes from patients challenged *in vivo* with allergen, but not irrelevant antigen, rapidly synthesize (within 4 h) mRNA for interferon-γ and produce immunoreactive interferon-γ. Interleukin-1α and interleukin-8 mRNA were also detected but showed no correlation with relevant antigen challenge. This study demonstrates that keratinocytes can produce interferon-γ and that this production is linked to challenge with relevant antigen in allergic contact dermatitis. These findings indicate that keratinocytes may amplify allergen-specific T-lymphocyte–triggered interferon-γ–dependent responses and might partially explain the speed of reaction in this common disease and other delayed hypersensitivity reactions involving the skin. ***Key words: cytokines/allergy/in situ hybridization/immunohistochemistry. J Invest Dermatol 106:1218–1223, 1996***

Allergic contact dermatitis results in the infiltration of mononuclear leukocytes including allergen-specific T lymphocytes (De Sousa and Parrott, 1969) that produce interferon-γ (Cher and Mosmann, 1987; Thomson *et al*, 1993). Keratinocytes constitutively express interferon-γ receptors (Nickoloff, 1987; Scheynius *et al*, 1992), and interferon-γ is known to induce keratinocyte cell surface MHC Class II antigen at sites of allergen challenge (Gawkrodger *et al*, 1987) on cultured keratinocytes (Basham *et al*, 1990) and in normal and inflamed skin *in vivo* (Griffiths *et al*, 1989). The cellular localization of interferon-γ production in allergic contact dermatitis, however, has not been demonstrated *in situ*. Using a combination of immunocytochemistry and *in situ* hybridization (ISH) for mRNA, we investigated the kinetics of the early production of interferon-γ, interleukin-1α (IL-1α), and IL-8 in the skin of a series of patients with known allergic contact dermatitis challenged with relevant allergen and irrelevant antigen.

MATERIALS AND METHODS

Subjects, Allergen Application, and Biopsies Sixteen patients (10 female and 6 male; age range, 25–77 yr) previously shown to be allergic to specific antigens, with a minimum of 2+ positivity at 48 h as assessed on the International Contact Dermatitis Research Group scale (Fregert, 1981), were studied. All were challenged with the relevant antigen in soft white paraffin and applied under 8-mm Finn chambers for up to 6 h. Six patients were also challenged with an irrelevant antigen. Up to four 6-mm–punch biopsies were taken from each patient from either the volar aspect of the forearm or the abdomen. Control biopsies were taken from either untreated skin or from patches where the vehicle alone was applied. Full details of the patients, biopsies, and allergens used are given in **Table I**. Further control specimens of normal skin were obtained from seven other subjects (age range, 30–54 yr; 6 female and 1 male; 5 from patients undergoing breast reduction and 2 forearm biopsies from volunteers). Approval of the study was obtained from the Lothian Medicine and Clinical Oncology Research Ethics Sub-Committee.

Tissue Processing The biopsies were bisected; half were placed into 10% buffered formalin, pH 7.4, and the other half were frozen in liquid nitrogen. Formalin-fixed biopsies were embedded in low-temperature paraffin wax, and the blocks were stored until use. Frozen biopsies were placed in OCT freezing compound (Miles Diagnostics, Elkhart, IN) and stored at −70°C until use.

Immunocytochemistry Cryostat sections (5 μ) were mounted on poly-L-lysine–coated slides, fixed in acetone, and stained with anti–interferon-γ antibodies (Chemicon 22/19 IgG_1 murine monoclonal at 1:500 dilution, and Genzyme rabbit polyclonal at 1:200 dilution) anti-CD3, or anti-CD1a antibodies (Dako, UK, Ltd.). This was followed by incubation with the appropriate biotinylated secondary antibody (Dako) in a standard ABC-alkaline phosphatase complex (Dako) method and development with Fast Red substrate (Sigma Chemical Co., St. Louis, MO). Controls for each run were performed using no primary antibody or irrelevant antibody of the same Ig isotype (monoclonal antibody) or species (polyclonal antibody). Stained epidermal cells were counted per millimeter of basement membrane using the Zeiss HOME microscope and IMPACT software.

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Reprint requests to: Dr. S. Howie, Department of Pathology, Edinburgh University Medical School, Teviot Place, Edinburgh EH8 9AG, UK.

Abbreviations: mRNA, messenger RNA; IL, interleukin; RNase, ribonuclease; ISH, *in situ* hybridization.

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Table I. Subject Details[a]

Patient No.	Gender	Age (yr)	Relevant Antigen	Irrelevant Antigen	Hour of Biopsy After Relevant Antigen	Hour of Biopsy After Irrelevant Antigen
1	Female	49	Nickel sulfate, 5%[b]		1 and 4	ND
2	Female	68	Nickel sulfate, 5%[b]		1 and 6	ND
3	Female	25	Nickel sulfate, 5%[b]		4	ND
4	Female	57	Nickel sulfate, 5%[b]	Fragrance mix, 8%[b]	1 and 4	4
5	Female	42	Nickel sulfate, 5%[b]		1, 4, and 6	ND
6	Female	42	Nickel sulfate, 5%[b]		1, 4, and 6	ND
7	Male	51	Nickel sulfate, 5%[b]		1 and 4	ND
8	Female	77	Wool alcohols mix, 30%[b]	Nickel sulfate, 5%[b]	4	1 and 4
9	Male	45	Thiuram mix, 1%[b]		1, 4, and 6	ND
10	Male	48	Paraphenyldiamine, 1%[b]	Nickel sulfate, 5%[b]	1, 4, and 6	4
11	Male	65	Bioban, 2.5%[c]	Nickel sulfate, 5%[b]	1 and 4	4
12	Male	48	4-Chloro-3-xylenol[b]	Nickel sulfate, 5%[b]	4	4
13	Male	65	Fragrance mix, 8%[b]		1 and 6	ND
14	Female	53	Nickel sulfate, 5%[b]		6	6
15	Female	27	Nickel sulfate, 5%[b]		6	ND
16	Female	48	Nickel sulfate, 5%[b]		6	ND

[a] All patients had control biopsies taken at time 0.
[b] European Standard Battery, Trolab, Biodiagnostics Ltd, Upton upon Severn, Worcestershire, UK.
[c] Oil and Cooling fluid series, Chemotechnique Diagnostics, Malmo, Sweden.

***In Situ* Hybridization** Cytokine mRNA was detected by ISH on 5-μm formalin-fixed sections using fluorescein isothiocyanate-conjugated oligonucleotide probes as described previously (Howie *et al*, 1992). Briefly, exon mixture [equimolar mixture of: for interferon-γ, antisense probes for exons 1 (29 bases), 2 (30 bases), and 4 (30 bases), catalog number BPR216; for IL-1α antisense probes for exons 5 (30 bases), 6 (30 bases), and 7 (31 bases), catalog number BPR4; for IL-8 antisense probes for the 5′ region (30 bases), the mid region (30 bases), and the 3′ region (31 bases), catalog number BPR100]; single interferon-γ exon 4 antisense, catalog number BPR215; and sense (30 bases), made to order. Oligonucleotide probes chemically synthesized and modified at the 5′ end with fluorescein isothiocyanate were purchased from R and D Systems Europe (Abingdon, Oxon, UK; sequence details available on request to the UK company or to R and D Systems, Minneapolis, MN). Sections of paraffin-embedded skin were cut onto 3-amino-propyltrimethoxysilane (SIGMA UK, Ltd.)-coated slides, dewaxed, rehydrated, treated with proteinase K (Boehringer Mannheim, UK Ltd.), prehybridized, hybridized overnight at 37°C, washed in standard saline citrate buffer, and incubated with affinity-purified (through protein G, Pharmacia UK Ltd, columns) rabbit anti-fluorescein isothiocyanate serum (Dako). All biopsies from an individual patient were hybridized in a single experiment. Immunohistochemical detection was carried out using a nitro blue tetrazolium 5-bromo-4-chloro-3-indolyl phosphate alkaline phosphatase substrate ISH detection kit from Dako. Negative controls with no probe were included in every experiment, and RNase pretreatment was carried out on a number of occasions before hybridization as extra negative controls. ISH reactions were scored "blindly" by two independent observers. Concordance was greater than 95%. Scoring was as previously described (Howie *et al*, 1992) on an arbitrary scale of 0 to 4, where 0 was negative and 4 was intensely positive.

Double Labeling for Interferon-γ mRNA and Cytokeratin Paraffin-embedded skin sections from unchallenged skin or skin challenged with relevant or irrelevant antigen were processed for interferon-γ mRNA as above and then incubated with murine monoclonal anti-pan-cytokeratin (Sigma C2562) at 1/50 dilution overnight at 4°C. After washing, sections were incubated (at room temperature) with anti-mouse IgG fluorescein isothiocyanate conjugate (Sigma F-2883) at 1/20 dilution for 40 min. After further washing, sections were mounted in Cityfluor (Cityfluor Ltd., City University London, UK) and viewed using a Leitz confocal fluorescence microscope. Controls included sections processed for interferon-γ mRNA and incubated with no primary monoclonal antibody and sections hybridized without a probe and then stained with monoclonal anti-pan-cytokeratin as above.

RESULTS

Immunocytochemical Localization of Interferon-γ and Specificity of Interferon-γ Immunocytochemistry Sections stained with either monoclonal or polyclonal antibody to interferon-γ after antigen challenge showed strong granular perinuclear cytoplasmic staining of many, but not all, keratinocytes as well as infiltrating inflammatory cells in the dermis **(Fig 1)**. No staining was detected in normal control biopsies **(Fig 1*a*)**, but strong staining was present 6 h after challenge with nickel as determined with either polyclonal rabbit anti–IFN-γ **(Fig 1*b*)** or monoclonal anti–IFN-γ **(Fig 1*c*)**.

Specificity for interferon-γ detection was checked by preblocking of sections with normal rabbit serum alone, with rabbit anti–IFN-γ, or with hyperimmune rabbit serum against a human lung antigen (the gift of Dr W Wallace, Dept. Pathology) at the same concentrations prior to incubation with murine monoclonal IgG2a isotype anti–IFN-γ antibody. Sections preblocked with normal rabbit serum or with the hyperimmune anti-lung antigen serum stained with the murine anti–IFN-γ monoclonal antibody (not shown), whereas staining was absent in those incubated with the rabbit anti–IFN-γ antibodies **(Fig 1*d*)**, indicating that both the monoclonal and polyclonal anti–IFN-γ antibodies recognized the same determinant in epidermal keratinocytes after challenge with relevant antigen. A negative control (no primary antibody) for the monoclonal antibody is shown in **Fig 1*e***. As a positive control, staining with monoclonal anti-CD1a (also IgG_{2a} isotype) is shown in **Fig 1*f***.

Quantification of Interferon-γ–Positive Cells in the Epidermis Three nickel-sensitive patients biopsied before and 6 h after challenge with nickel sulfate and five normal skin biopsies were evaluated for the numbers of labeled epidermal cells/mm basement membrane after staining of parallel sections with anti–IFN-γ, anti-CD3, or anti-CD1a. The results **(Table II)** show no significant differences in numbers of CD3-positive or CD1a-positive cells between normal skin, control biopsies from allergic patients, and 6-h biopsies from antigen-challenged patients allergic to nickel. Control biopsies from the patients, however, had significantly higher numbers of anti–IFN-γ-reactive epidermal cells than normal skin ($p < 0.02$, Wilcoxon Mann-Whitney test). After 6 h of challenge with nickel sulfate, there was a large increase in the numbers of anti–IFN-γ-labeled epidermal cells, indicating very rapid synthesis of anti–IFN-γ-immunoreactive protein ($p < 0.05$, Wilcoxon Mann-Whitney test).

ISH for Interleukin 1α, Interleukin 8, and Interferon-γ mRNA The cytoplasmic staining of keratinocytes with anti–IFN-γ could be due to either internalization of exogenously produced material or keratinocyte synthesis of the protein. To distinguish between these possibilities, we looked for evidence of transcription of interferon-γ mRNA in the keratinocytes of patients challenged with relevant or irrelevant antigen. ISH was carried out

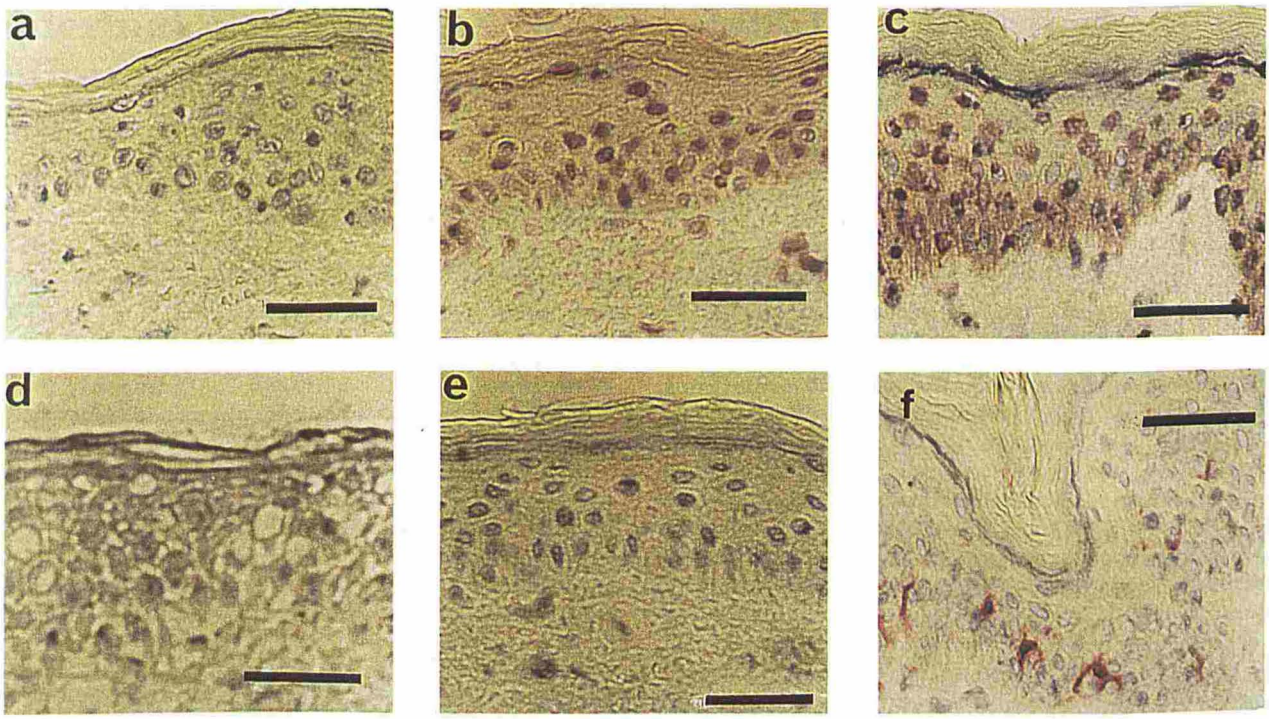

Figure 1. Immunohistochemical staining for interferon-γ in normal and nickel sensitive challenged skin. Biopsies from normal unchallenged skin and nickel-sensitive skin 6 h after nickel sulfate challenge are shown. Normal skin does not stain for interferon-γ (*a*). A punch biopsy from a nickel-sensitive patient taken after challenge shows that keratinocytes stain with both polyclonal rabbit anti–IFN-γ (*b*) and monoclonal (isotype IgG_{2a}) anti–IFN-γ (*c*). The staining of monoclonal anti–IFN-γ is blocked by preincubation with the polyclonal antibody (*d*). The negative control with no primary antibody is shown in *e*, and a positive isotype control monoclonal, anti-CD1a (IgG_{2a}), is shown in *f*. *Scale bar*, 50 μm.

for IL-1α, IL-8, and interferon-γ using oligonucleotide antisense exon probe mixtures. All the probes were used on all the biopsies from the same patient in the same run. **Figure 2*a*** shows that staining for interferon-γ mRNA in the biopsy taken at time 0 is absent with the exon mixture probe. **Figure 2*b*** shows staining with the same probe in the biopsy taken 6 h after challenge with relevant antigen (5% nickel sulfate), and **Fig 2*c*** shows that a parallel section from the same biopsy is unstained with no probe. **Figures 2*d*** through **2*f*** show the same biopsy 6 h after allergen is labeled when stained with the antisense exon 4 probe only **(Fig 2*d*)** but negative when stained with either the sense probe for exon 4 **(Fig 2*e*)** or the antisense probe applied after RNase pretreatment of the section **(Fig 2*f*)**. These results indicate that epidermal cells with all the morphological characteristics of keratinocytes did indeed synthesize interferon-γ mRNA after challenge with a relevant allergen.

Interferon-γ mRNA-Positive Cells Are Keratinocytes To ensure that the cells stained for interferon-γ mRNA by ISH were indeed keratinocytes, a double ISH and immunohistochemical procedure was carried out. Sections from biopsies of relevant antigen challenge were double-stained for interferon-γ mRNA and cytokeratin. Control sections leaving out the probe (ISH) or the anti-cytokeratin antibody (immunostaining) were negative for the ISH reaction or fluorescence, respectively. A double-stained section is shown in **Fig 3** and illustrates that epidermal cells were simultaneously positive for both interferon-γ mRNA and cytokeratin.

Kinetics of Interferon-γ mRNA Production, But Not IL-1α or IL-8, Correlate with Allergen Challenge Only To examine the kinetics of mRNA production, after staining with the exon

Table II. Number of Reactive Epidermal Cells per Millimeter of Basement Membrane[a]

	CD3+		CD1a+		Interferon γ +	
Source of Biopsy	No Treatment	$NiSO_4$, 5%[b]	No Treatment	$NiSO_4$, 5%[b]	No Treatment	$NiSO_4$, 5%[b]
Normal 1	<1	ND[c]	9	ND[c]	2	ND[c]
Normal 2	1	ND[c]	8	ND[c]	3	ND[c]
Normal 3	1	ND[c]	10	ND[c]	<1	ND[c]
Normal 4	<1	ND[c]	9	ND[c]	<1	ND[c]
Normal 5	<1	ND[c]	9	ND[c]	5	ND[c]
P14, nickel-sensitive	<1	<1	10	13	20	112
P15, nickel-sensitive	1	1	12	12	7	67
P16, nickel-sensitive	5	1	9	9	44	147

[a] Consecutive sections from 6-mm biopsies from five nonallergic untreated controls and from three nickel-sensitive patients before (No treatment) and 6 h after challenge with relevant antigen ($NiSO_4$, 5%) were stained with anti-CD3, anti-CD1a, or anti-interferon γ monoclonal antibodies. Labeled cells were counted per millimeter of basement membrane along the whole length of each section.

[b] European Standard Battery, Trolab, Biodiagnostics Ltd, Upton upon Severn, Worcestershire, UK; 6-h exposure.

[c] ND, not done.

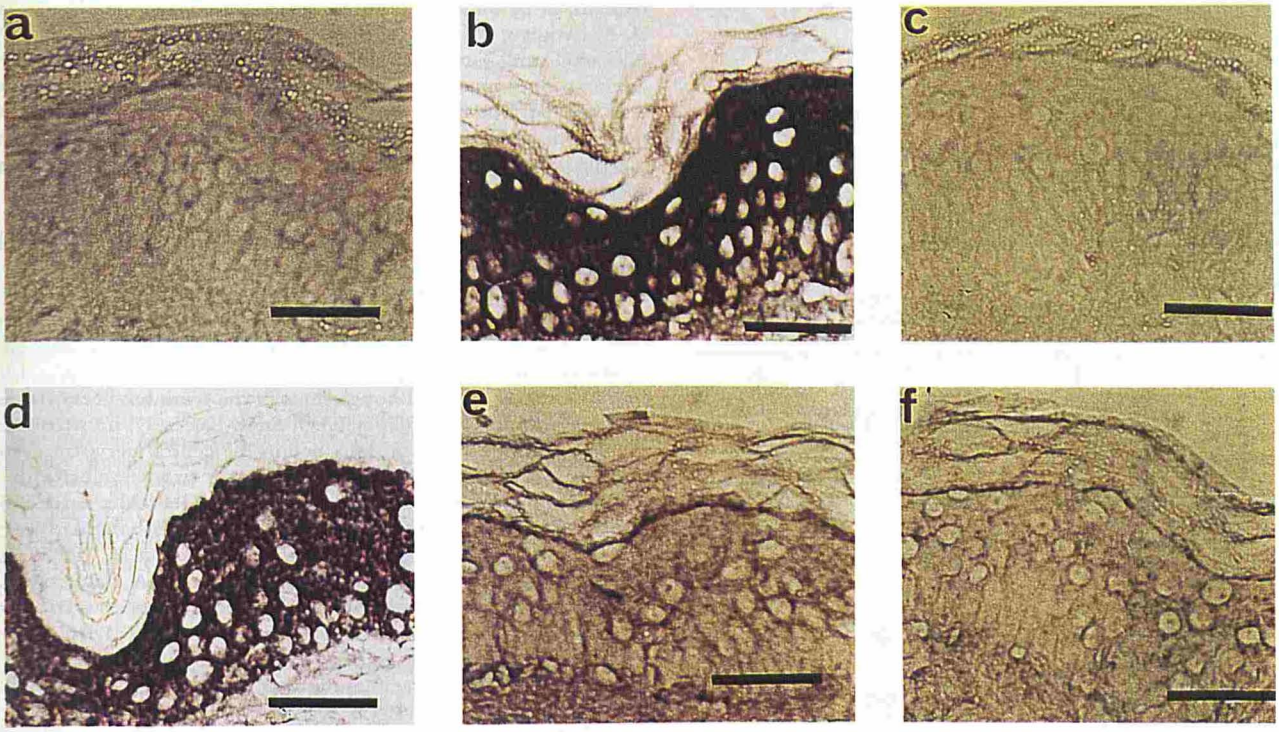

Figure 2. ISH for interferon-γ in nickel-sensitive skin before and after challenge. ISH for cytokine mRNA was carried out as described in detail previously (Howie *et al*, 1992). Shown are sections from a nickel-sensitive patient before and 6 h after application of nickel sulfate. The control (time 0) biopsy is negative with the interferon-γ anti-sense oligonucleotide exon mixture probe (equimolar mixture of probes for exons 1, 2, and 4) (*a*). After 6-h application of 5% nickel sulfate, the keratinocytes are strongly positive with the exon mixture probe (*b*), negative with no probe (*c*), positive with the probe for exon 4 only (*d*), and negative with both the sense probe for exon 4 (*e*), and the anti-sense probe for exon 4 applied after RNase digestion (*f*). *Scale bar*, 50 μm.

probe mixtures for interferon-γ, IL-1α, or IL-8 mRNA, the sections were coded and assessed "blindly" by two independent observers. IL-8 mRNA was detected even in epidermal cells of untreated skin, and both IL-1α and IL-8 mRNA were detected in epidermal keratinocytes of skin challenged with irrelevant or relevant antigen **(Fig 4)**. Detection of interferon-γ mRNA in keratinocytes showed a statistically significant correlation ($p < 0.001$ at 1 and 4 h; $p < 0.02$ at 6 h) with challenge with relevant antigen compared with the control biopsies from the same patients **(Fig 4)**. Comparison of the biopsies challenged with relevant antigen to those challenged with irrelevant antigen after 4 h also showed a significant difference ($p < 0.04$, Wilcoxon-Mann-Whitney test).

It should be noted that two out of three time 0 biopsies from the patients used to enumerate interferon-γ protein-containing epidermal cells **(Table II)** were negative for interferon-γ mRNA, and that the third patient's biopsy had a score of 1 at time 0. The biopsies from these three patients 6 h after allergen challenge had mRNA scores of 3, 4, and 3, respectively.

DISCUSSION

These results demonstrate that the early phase of allergic contact dermatitis is characterized *in situ* by the rapid appearance of interferon-γ protein and mRNA in keratinocytes as well as in infiltrating lymphocytes. In addition, in three patients tested for both protein and mRNA expression there was discordance between protein and mRNA levels in the baseline time 0 biopsies, as mRNA was only detected in one of three patients, whereas all three had significantly higher numbers of epidermal interferon-γ protein-containing cells per millimeter of basement membrane than did nonallergic normal controls. This may reflect stored protein in the absence of active mRNA transcription and may indicate that the "resting" epidermis of allergic individuals is primed to respond

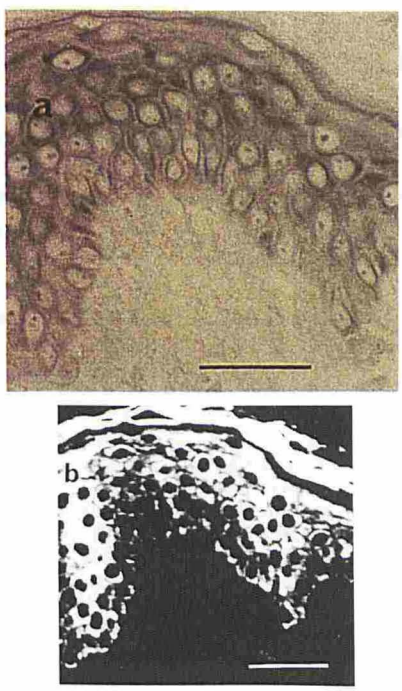

Figure 3. Interferon-γ mRNA is found in keratin-positive cells. Double immunofluorescence/ISH staining of a section from a biopsy of nickel-sensitive skin taken 6 h after application of 5% nickel sulfate. *a*, light microscope appearance of a positive ISH using the interferon-γ anti-sense exon 4 probe; *b*, confocal laser scanning microscopic appearance of the same section showing positive cytokeratin staining. *Scale bar*, 50 μm.

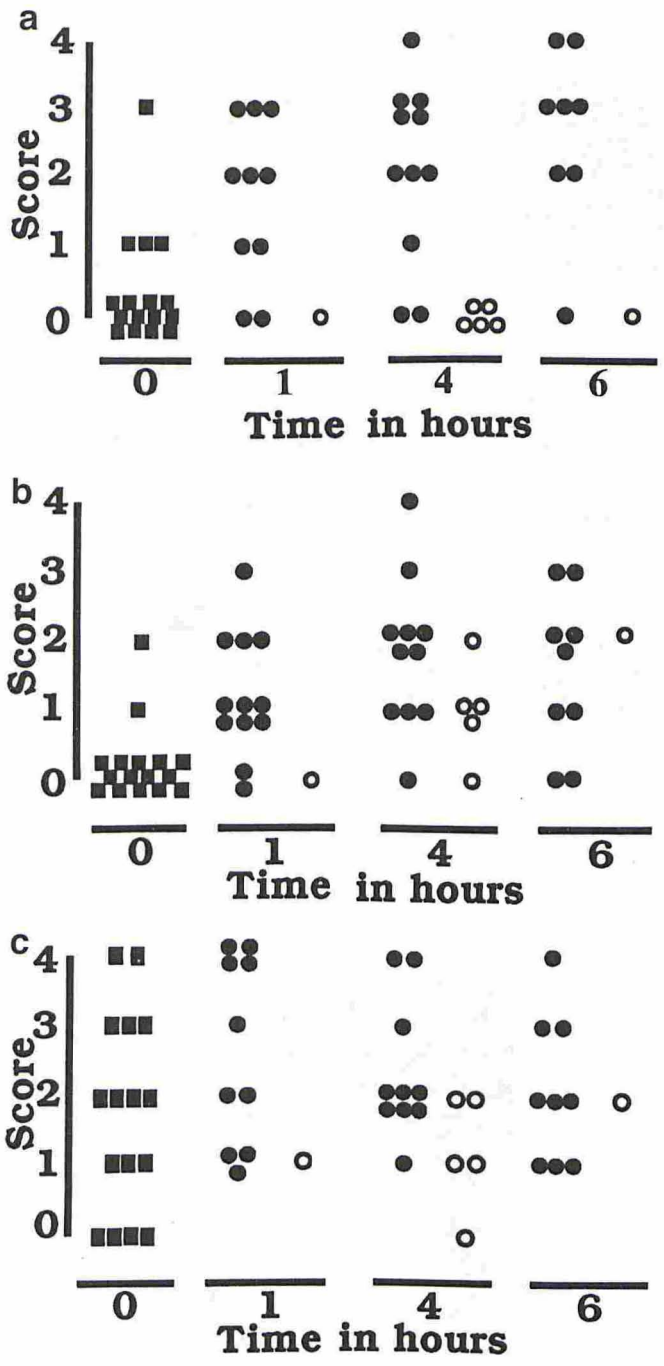

Figure 4. ISH scores for interferon-γ, IL-1α, and IL-8 in biopsies from allergic patients before and after challenge with relevant or irrelevant antigen. ISH scores of sections from patient biopsies at 1, 4, and 6 h after challenge with relevant (●) or nonrelevant (○) antigen, and from nonchallenged skin of the same patient (■). Staining was scored blindly on an arbitrary scale from 0 (no staining) to 4 (strongly labeled). Results are given for interferon-γ (*a*), IL-1α (*b*), and IL-8 (*c*).

quickly to a relevant allergic stimulus. Production of other inflammatory cytokines, IL-1α and IL-8, is also demonstrable in keratinocytes and infiltrating leukocytes, but as these were also produced in response to challenge with irrelevant antigen and in some cases to vehicle alone, they are not specific markers of an allergic reaction.

Previously, keratinocytes have been shown to constitutively contain, or be induced to produce, cytokines, including IL-1α and IL-8 (Barker *et al*, 1990; McKay and Leigh, 1991; Chu *et al*, 1992; Nickoloff and Turka, 1993), but the finding that keratinocytes could produce interferon-γ in response to relevant contact antigens was unexpected. Interestingly, Auböck *et al* (1985)[1] reported the immunohistochemical detection of interferon-γ protein in keratinocytes in human inflammatory dermatoses, but the kinetics and specificity of production was not studied nor was the presence of interferon-γ mRNA.

Our findings of interferon-γ mRNA production by human keratinocytes after relevant antigen challenge are in contrast with findings published in Balb/c murine skin (Enk and Katz, 1992), which may reflect species differences between mouse and human skin. It is accepted in the literature that interferon-γ is produced by T lymphocytes and natural killer cells (Kasahara *et al*, 1983; Munakata *et al*, 1985), although most of the work has been done in tissue culture using lymphocyte cell lines. Recently, immunoreactive interferon-γ has been described in the central nervous system (Kiefer *et al*, 1991; Olsson *et al*, 1994), stromal elements of lymphoid tissue, and vascular endothelium (Heufelder and Bahn, 1993). Interferon-γ mRNA has been detected in B-lymphocyte cell lines (Dayton *et al*, 1992) and fibroblasts (Rady *et al*, 1995), and there are several reports of interferon-γ mRNA detection by polymerase chain reaction in nonlymphoid tissues, which the investigators have ascribed to contamination by T lymphocytes (Barker *et al* 1991; Dallman *et al*, 1991; Halloran, 1993). It was of particular interest that the immunoreactive interferon-γ reported in neurons (Kiefer *et al*, 1991; Olsson *et al*, 1994) reacted with anti–IFN-γ antibodies, behaved biologically as interferon-γ, and bound to its receptor (Olsson *et al*, 1994) but had a different molecular weight from that of the conventional molecule. In addition, this protein is the product of a different gene and is not a product of interferon-γ mRNA, as no mRNA was detected by polymerase chain reaction in neurones positive for the protein (Kiefer *et al*, 1991). Polymerase chain reaction investigation of the skin has also been reported to show upregulation in both epidermal and dermal interferon-γ mRNA production after repeated tape stripping (Nickoloff and Naidu, 1994). While polymerase chain reaction detection of cytokine mRNAs is a powerful tool, it does not allow definition of the types of cells synthesizing interferon-γ in tissues undergoing inflammatory reactions, in which cells of several different lineages may be involved. For this reason, and to determine whether we were detecting an interferon-γ mimic (as found in neurones) or the conventional cytokine, we investigated the production of interferon-γ in allergic contact dermatitis by ISH for mRNA. This approach allowed us to demonstrate that allergen challenge, but not irrelevant antigen challenge, was characterized by keratinocyte as well as lymphocyte mRNA transcription and synthesis of interferon-γ protein. As keratinocytes express interferon-γ receptors (Nickoloff, 1987; Scheynius *et al*, 1992), and because interferon-γ protein is known to upregulate its own mRNA production (Cockfield *et al*, 1993), control of keratinocyte synthesis of the cytokine may be a consequence of local specific allergen-reactive T-cell production of the cytokine, which binds to the keratinocyte receptors for interferon-γ. We postulate that this increased interferon-γ protein synthesis induced by both paracrine (allergen-specific T lymphocytes) and autocrine (keratinocytes) mechanisms acts as an amplification loop at sites of allergen exposure. This helps to explain the rapidity and degree of local inflammation seen in the challenge reaction of allergic contact dermatitis.

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[1] Auböck J, Romani N, Sifter M, Fritsch P: Interferon-gamma in human skin. *J Invest Dermatol* 84:456, 1985 (abstr.).

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